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Correlation between *Plasmodium yoelii nigeriensis* Susceptibility to Artemisinin and Alkylation of Heme by the Drug

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Evidence of artemisinin (ART) resistance in all of the Greater Mekong Region is currently of major concern. Understanding of the mechanisms of resistance developed by *Plasmodium* against artemisinin and its derivatives is urgently needed. We here demonstrated that ART was able to alkylate heme in mice infected by the ART-susceptible strain of *Plasmodium yoelii nigeriensis*, Y-control. After long-term drug pressure, the parasite strain (Y-ART3) was 5-fold less susceptible to ART than Y-control. In the blood of mice infected by Y-ART3, no heme-artemisinin adducts could be detected. After release of ART drug pressure, the parasite strain obtained (Y-REL) regained both drug susceptibility to ART and increased ability to produce covalent heme-artemisinin adducts. The correlation between parasite ART susceptibility and alkylation of heme by the drug confirms that heme or hemozoin metabolism is a key target for efficacy of ART as an antimalarial.

Artemisinin (ART) is a natural drug containing a biologically relevant 1,2,4-trioxane structure. Artemisinin-based combination therapies (ACT) are recommended by the WHO as the first-line treatment for malaria caused by *Plasmodium falciparum*, the most deadly species that infects humans, and countries where malaria is endemic have progressively shifted their national treatment policies accordingly (1). However, emergence of *P. falciparum* field isolates with disturbing levels of ART resistance has now been reported in several Southeast Asian countries (2–5). To circumvent resistance and to develop new drugs based on a peroxide structure, identification of the key features of ART activity and *Plasmodium* ART resistance mechanisms is necessary. The reactivity of the peroxide function of ART in the presence of iron(II)-heme, which is a flat and achiral molecule, is out of the classical "key and lock" paradigm for drugs. In fact, the iron(II)-mediated reductive activation of the peroxide function generates a short-lived alkoxy radical, which quickly rearranges to a C-centered primary radical that alkylates heme via an intramolecular process to produce covalent heme-drug adducts (6–8). These heme-artemisinin adducts have been evidenced in malaria-infected mice (9). As a consequence of heme alkylation, the accumulation of nonpolymerizable redox-active heme derivatives is expected to be toxic for the parasite (10).

We developed a model of a malaria parasite resistant to ART and its derivatives to study mechanisms of *Plasmodium* resistance. The multidrug-resistant strain of the rodent-derived *Plasmodium yoelii nigeriensis*, namely, Y-ART3, had so been selected by continuous ART drug pressure for more than 5 years *in vivo* (11). The susceptibility of this laboratory strain of *P. yoelii* Y-ART3 to ART decreased by a factor of 5 compared to its parental ART-sensitive strain (Y-control). It was significant that the Y-ART3 strain led to much lower content of hemozoin in the parasites (41 times less hemozoin in Y-ART3 parasites than in Y-control parasites). Interestingly, after release of the drug pressure, the 50% inhibitory concentrations (IC_{50}s) and hemozoin content of strain Y-REL readily returned to the levels of the Y-control strain (11).

In the present work, we compared the levels of alkylation of heme by ART within mice infected by *Plasmodium yoelii nigeriensis* with different levels of susceptibility to ART (namely the Y-control, Y-ART3, and Y-REL strains). In fact, the reduced susceptibility to ART and impairment of heme/hemozoin metabolism were correlated with a significant decrease in heme alkylation by ART.

MATERIALS AND METHODS

Treatment of mice. Swiss female albino mice (25 to 30 g) were inoculated by the intraperitoneal (i.p.) route, with 2×10^7 erythrocytes parasitized by *P. yoelii nigeriensis*. The strains Y-ART and Y-REL were selected after long-term *in vivo* ART pressure and after release of drug pressure, respectively, as previously described (11). At least 3 independent experiments were conducted for each strain. A total of 7 mice were inoculated with the wild-type strain (Y-control), 9 were inoculated with the ART-resistant strain (Y-ART3), and 8 were inoculated with the strain that regained ART sensitivity after release of the drug pressure (Y-REL). Infected mice were treated by the i.p. route with a single dose of ART (100 mg/kg of body weight diluted in 100 μl of dimethyl sulfoxide [DMSO]). As control experiments, 3 healthy mice also received the same dose of ART under the same conditions. Mice were sacrificed 5.5 ± 1 h after treatment. The spleen and blood from retro-orbital puncture were collected and analyzed individually. Urine was collected by groups of 2 to 3 mice during the hours following treatment. Urine and spleen were kept at −20°C until analyses.

Treatment of blood. Mouse blood was collected by retro-orbital puncture in heparinized tubes. Blood was washed with phosphate-buffered saline and centrifuged at 3,000 rpm for 5 min. Blood pellet was treated with saponin at 0.025% for 5 min at 0°C (protocol modified from reference 12). The mixture was then centrifuged at 3,000 rpm for 10 min. The pellet containing parasites and some membrane residues was then conserved at −20°C until analyses. For liquid chromatography-mass spectrometry (LC-MS) analysis, the blood pellet was homogenized by vortex and sonicated. An aliquot was withdrawn (100 μl), diluted with acetic acid (200 μl), and precipitated by addition of acetone (750 μl). The mixture was centrifuged at 4,000 rpm for 30 min, and the supernatant

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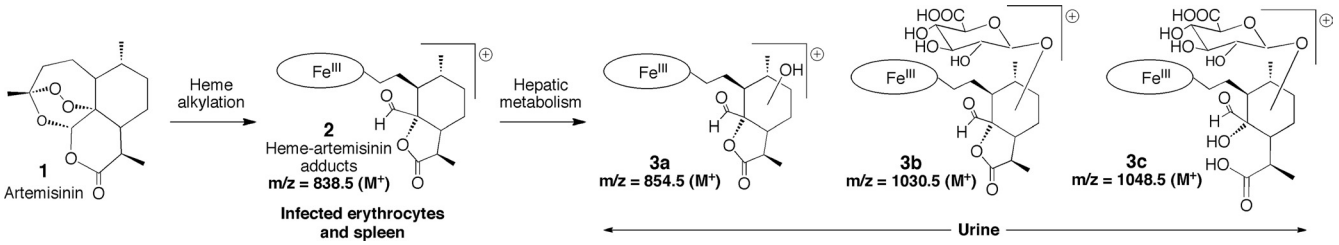

FIG 1 Structures of artemisinin (diagram 1) and of the heme-artemisinin adducts detected in the blood pellets and spleens (diagram 2) or in the urine samples (diagrams 3a to c).

solution was withdrawn, concentrated at room temperature, and analyzed by LC-MS.

Spleen treatment. Each whole mouse spleen was crushed with sand in a mortar and then extracted with glacial acetic acid (1.5 ml). An aliquot (160 μl) of the acetic extract was withdrawn, and the macromolecules were then precipitated by slow addition of acetone (650 μl) and centrifuged at 4,000 rpm for 30 min. The supernatant solution, containing heme derivatives, was withdrawn, concentrated at room temperature, and analyzed by LC-MS in order to detect covalent adducts resulting from the alkylation of heme by ART.

Urine treatment. Urine was collected and analyzed by lots of 2 to 3 mice without extra treatment. Urine was centrifuged at 4,500 rpm for 10 min. The supernatant phase was analyzed by LC-MS.

LC-MS analyses. Analytical separations were performed on a Waters 5-μm C_{18} X-bridge column (100 by 4.6 mm) equipped with a 5-μm C_{18} precolumn. Compounds were eluted under the following conditions: solvent (A), methanol-water-formic acid, 60/40/1; solvent (B), methanol-water-formic acid, 80/20/1; gradient from A/B = 100/0 to A/B = 0/100 over 10 min, followed by 10 min at A/B = 0/100. The elution rate was 0.8 ml/min, and the UV/visible detection was performed at 406 nm. The injected volume was 100 μl. The retention times were 8.4 min for heme and 10.3 to 12.1 min for heme-artemisinin adducts (several peaks due to regioisomers) (9). Positive-ion electrospray mass spectrometry was performed on a Q-Trap AB Sciex quadrupole instrument in scan mode.

When possible, each acetic acid extract or urine sample was treated and analyzed several times. Analyses were classified by a "yes or no" scheme. The adduct was considered present when (i) the *m/z* value was detected with the expected isotopic pattern (M-2 due to Fe^{54}) and (ii) the extracted ionic current (XIC) exhibited a signal/noise ratio higher than 3.

Statistical analysis. All tests were performed using the SigmaStat 2.03 statistical program (SigmaStat, Heame Scientific Software, Chicago, IL). These data were analyzed using the Fisher test. A comparison was considered statistically significant if the *P* value was ≤0.05.

Animal procedures. All procedures involving living animals fully conformed to French and European regulations (EEC directive 86/609 dated 24 November 1986 and modified by the new regulations 2010/63/UE). All animal manipulations were carried out in the animal room of the Parasitology Department of Rangueil Hospital, Toulouse, which has been placed under the control of the National Veterinary Services (agreement B31 555 03). The staff in charge of the animal experiments has received the appropriate training and has been granted a license delivered by the French Agricultural Ministry for experimentation on small laboratory animals. These *in vivo* studies were approved by the French Institutional Animal Experimentation Ethics Committee (approval no. MP/R/05/32/11/07 and MP/R/06/33/11/07).

RESULTS AND DISCUSSION

The spleen, urine, and blood from malaria-infected mice (Y-control, Y-ART3, or Y-REL of *P. yoelii nigeriensis*) and ART-treated (100 mg/kg) mice compared to control uninfected mice were analyzed by LC-MS in order to detect covalent adducts resulting from the alkylation of heme by ART.

The heme-artemisinin adducts were expected at *m/z* 838.5 in the spleen and in the parasite pellets from blood of mice. Conversely, the metabolized adducts were expected at *m/z* 854.5, 1,030.5, and/or 1,048.5 in the urine; the structures are shown in Fig. 1 (9). The adduct was considered present when (i) the *m/z* value was detected with the expected isotopic pattern (M-2 due to Fe^{54}) and (ii) the extracted ionic current (XIC) exhibited a signal/noise ratio higher than 3. Results are reported in Table 1.

In the spleens and urine samples from mice infected by the Y-control strain, heme-artemisinin adducts were detected in 86% and 66% of analyses, respectively. In mice infected with the Y-ART3 strain, with a reduced susceptibility to ART, alkylation of heme was detected in only 50% of analyses, both in the spleens and in urine samples. After release of the pressure and recover of drug susceptibility (strain Y-REL), heme-drug adducts were detected in 100% of analyses, close to the result obtained with the Y-control strain. Heme-artemisinin adducts were not detected in the spleens and urine samples from healthy mice treated under the same conditions. Therefore, alkylation of heme by ART was associated with parasite drug susceptibility. In spleens and urine samples of mice infected with Y-ART3, heme-drug adducts were detected in fewer samples than in spleens and urine samples from mice infected with the Y-control or Y-REL strain. However, they were still detectable in 50% of samples from mice infected with Y-ART3. Indeed it should be noted that under drug pressure, the decrease of susceptibility of the strain Y-ART3 to ART was only moderate, the *ex vivo* IC_{50} on the Y-ART3 parasite being only 5-fold that observed for the Y-control strain (0.17 ± 0.04 versus 0.86 ± 0.11) (11).

The correlation between heme alkylation and ART parasite sensitivity was even much more significant in the blood pellets. It should be emphasized that in mice infected with the ART-sensitive Y-control and Y-REL strains, alkylation of heme statistically occurred at detectable levels in 83 and 100% of analyses, respectively, whereas it could be detected in none of the blood parasite pellets of mice infected by the ART-resistant Y-ART3 strain (Y-

TABLE 1 Detection of heme-artemisinin adducts in blood pellets, spleens and urine samples from mice infected with the Y-control, Y-ART3, and Y-REL strains of *P. yoelii*

Infection group (no. of mice)	No. (%) of samples with adducts/total		
	Blood pellets	Spleen extracts	Urine
Y-control (7)	5/6 (83)	6/7 (86)	2/3 (66)
Y-ART3 (9)	0/5 (0)	4/8 (50)	2/4 (50)
Y-REL (8)	2/2 (100)	8/8 (100)	2/2 (100)
Healthy, uninfected (3)	0/3 (0)	0/3 (0)	0/3 (0)

control versus Y-ART3, $P = 0.015$; Y-control versus Y-ART3, $P = 0.048$). We can also notice that results obtained in parasite pellets with Y-control and Y-REL were statistically comparable ($P = 1$).

As alkylation of heme takes place into the intraerythrocytic parasites, the most relevant analysis is obviously that obtained from the blood parasite pellets. Further accumulation, metabolism, and disposition of these adducts is a dynamic process. Therefore, their distribution may be responsible for quantitatively different results between the blood pellets and the spleens on one hand and urine samples on the other. In addition, the workup required prior to analysis may explain quantification differences between blood pellets and spleen extracts.

Plasmodium yoelii nigeriensis sensitivity to ART is thus linked to the presence of heme-artemisinin adducts, whereas the loss of malaria parasite susceptibility to ART is associated with significant decrease in the quantities of heme-drug adducts. Therefore, the present study definitely shows that ART activity against *Plasmodium* is correlated to heme metabolism via production of heme-artemisinin adducts. We have previously demonstrated that *Plasmodium yoelii nigeriensis* resistance to artemisinin derivatives was linked to major modifications in the parasites' heme metabolism, with a loss of hemozoin formation linked to the down-expression of the heme detoxification protein (HDP) (11). Moreover, Klonis et al. also showed that artemisinin activity was dependent on hemoglobin digestion by the parasite since inhibition of hemoglobin degradation pathways significantly decreases artemisinin sensitivity (13). We also showed that artemisinin-resistant selected strains were able to detoxify the free heme by an alternative catabolism pathway involving glutathione (GSH) mediation (11). Taken altogether, these results suggest that ART acts by several pathways involving heme catabolism and redox mechanisms.

Conclusion. We here have demonstrated a clear association between (i) *Plasmodium yoelii nigeriensis* susceptibility to artemisinin or (ii) antimalarial efficiency of ART on one side and the alkylating property of the drug on the other. This suggests that the alkylating reactivity may be one of the key factors of the antimalarial activity.

The exact role of heme-artemisinin adducts in the death of the parasite is still largely debated. However, heme is a master piece of the mechanism of action of endoperoxide-containing antimalarial drugs. These data should be taken into account in the future development of antiplasmodial drug combinations to propose ACTs also containing drugs aimed at parasite targets other than heme.

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